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Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2005

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT	($) 500

Complete if Known	
Application Number	09/891,138
Filing Date	June 25, 2001
First Named Inventor	Lin, Daniel Chi-Hong
Examiner Name	Christopher J. Nichols
Art Unit	1647
Attorney Docket No.	018781-006210US

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☒ Deposit Account Deposit Account Number: 20-1430 Deposit Account Name: Townsend and Townsend and Crew LLP

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

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FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

Application Type	FILING FEES Fee ($)	FILING FEES Small Entity Fee ($)	SEARCH FEES Fee ($)	SEARCH FEES Small Entity Fee ($)	EXAMINATION FEES Fee ($)	EXAMINATION FEES Small Entity Fee ($)	Fees Paid ($)
Utility	300	150	500	250	200	100	
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 or, for Reissues, each claim over 20 and more than in the original patent	50	25
Each independent claim over 3 or, for Reissues, each independent claim more than in the original patent	200	100
Multiple dependent claims	360	180

Total Claims	Extra Claims	Fee ($)	Fee Paid ($)
_____	-20 or HP = _____	x _____	= _____

HP = highest number of total claims paid for, if greater than 20

Indep. Claims	Extra Claims	Fee ($)	Fee Paid ($)
_____	-3 or HP = _____	x _____	= _____

HP = highest number of independent claims paid for, if greater than 3

Multiple Dependent Claims Fee ($)	Multiple Dependent Claims Fee Paid ($)
_____	_____

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof	Fee ($)	Fee Paid ($)
_____ - 100 =	_____ / 50 =	_____ (round up to a whole number) x	_____	= _____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	_____
Other: **Filing a brief in support of an appeal**	**500**

SUBMITTED BY

Signature		Registration No. (Attorney/Agent) 44,879	Telephone 415-576-0200
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v

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On 11 March 2005

TOWNSEND and TOWNSEND and CREW LLP

By: Malinda Adagist

PATENT
Attorney Docket No.: 018781-006210US
Client Ref. No.: T00-013-1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re application of:	Confirmation No. 8826
Daniel Chi-Hong Lin	Examiner: Christopher J. Nichols
Application No.: 09/891,138	Group Art Unit: 1647
Filed: June 25, 2001	APPEAL BRIEF UNDER 37 C.F.R. § 41.37
For: NOVEL RECEPTORS	
Customer No.: 20350	

Mail Stop Appeal Brief-Patents
Commissioner for Patents
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Sir:

This Appeal Brief is submitted pursuant to 37 C.F.R. § 41.37, following the Notice of Appeal filed on August 11, 2004. Also submitted with this Brief is authorization to pay the fee as set forth in 37 C.F.R. §41.20(b)(2) and the fee authorization for a five-month extension of time.

TABLE OF CONTENTS

I. REAL PARTY IN INTEREST

The real party in interest in U.S. Application No. 09/891,138 is Amgen, Inc.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals, interferences, or judicial proceedings at this time.

III. STATUS OF THE CLAIMS

Claims 1, 2, 4, 5, 8-12, 14-29, and 32-67 are cancelled.

Claims 3, 6, 7, 13, 30, and 31 are rejected.

Claims 3, 6, 7, 13, 30, and 31 are being appealed.

IV. STATUS OF AMENDMENTS

Appellant filed an Amendment on August 11, 2004 with an accompanying Declaration under 37 C.F.R. § 1.132 by Daniel Lin, Ph.D. that was subsequent to a final rejection. The Advisory Action mailed September 7, 2004 states that the Response and Amendment filed August 11, 2004 was entered in full. The Advisory Action also states that the Declaration was received and taken into consideration.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Summaries of the subject matter of independent claims 3, 6, and 13 are provided below. For convenience, Appellant has also included summaries for dependent claims 7, 30, and 31.

A. Claim 3

The subject matter recited in claim 3 relates to an isolated nucleic encoding a mouse TGR18 polypeptide comprising at least 200 contiguous amino acids of SEQ ID NO:2. SEQ ID NO:2 is set forth on page 58 of the specification. This claim is further supported in the specification, *e.g.*, at page 6, lines 1-2, which states that the invention provides nucleic acids encoding novel G-protein-coupled receptors (GPCRs); and page 12, lines 11-16, which teaches that a "GPCR" of the invention refers to polymorphic variants, alleles, mutants, and interspecies

homologs and GPCR domains that share identity over a window of amino acids. At page 18, lines 8-10 and lines 29-30, the specifications additionally teaches that two or more polypeptide sequences can be the same over a specified region (lines 8-10) and that a region can be 200 contiguous positions of a sequence (lines 29-30).

B. Claim 6

The subject matter recited in claim 6 relates to an isolated nucleic acid encoding a polypeptide comprising an amino acid sequence of SEQ ID NO:2. The claim is further supported in the specification, *e.g.*, at pages 58 and 59, which provide mouse TGR18 DNA and protein sequences. The specification additionally defines the claim at page 2, lines 1-2, and page 3, lines 28-30, which teaches that the invention provides nucleic acids that encode novel G protein coupled receptors, and that a polypeptide of the invention can comprise an amino acid sequence of SEQ ID NO:2, respectively.

C. Claim 7

The subject matter recited in dependent claim 7 relates to an isolated nucleic acid, wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO:1. The claim is further supported in the specification, *e.g.*, at page 3, lines 11-13.

D. Claim 13

The subject matter recited in claim 13 relates to an isolated nucleic acid encoding a G-protein coupled receptor polypeptide that transduces an increase in intracellular calcium, wherein the nucleic acid encodes a polypeptide comprising 95% or greater amino acid identity to the amino acid sequence of SEQ ID NO:2. The claim is further supported in the specification, *e.g.*, at page 11, lines 11-15, which teaches that a GPCR of the invention can have 95% or higher amino acid sequence identity to SEQ ID NO:2, and, *e.g.*, at page 11, lines 4-10 which teaches that GPCR activity can be measured by assessing intracellular calcium levels.

E. Claim 30

The subject matter recited in claim 30 relates to an expression vector comprising the nucleic acid of any one of claims 3, 6, 7, or 13. The claim is further supported in the specification, *e.g.*, at page 4, lines 5-7, which teaches that the invention provides expression vectors comprising the nucleic acids of the invention.

F. Claim 31

The subject matter recited in claim 31 relates to a host cell transfected with the vector of claim 30. The claim is further supported in the specification, *e.g.*, at page 4, lines 5-7, which teaches that the invention provides host cells comprising the expression vectors.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 3, 6, 7, 13, 30, and 31 stand rejected under 35 U.S.C. § 101 as allegedly lacking utility.

Claims 3, 6, 7, 13, 30, and 31 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement in view of the alleged lack of utility. Claims regarding variants and fragments of SEQ ID NO:1 are additionally rejected under 35 U.S.C. § 112, first paragraph, for alleged lack of enablement.

Claims 3, 6, 7, 13, 30, and 31 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking adequate written descriptive support.

VII. ARGUMENT

A. Rejection under 35 U.S.C. § 101 utility

1. Standards to Assess Utility

To satisfy the requirements of 35 U.S.C. § 101, an applicant must claim an invention that is "useful." In the following sections, Appellant demonstrates that the claimed invention satisfies the requirement for utility.

According to MPEP §2107, the Examiner should review the claims and the supporting written description to determine whether the utility requirement under 35 U.S.C. §101 is met. No rejection based on lack of utility should be made if an invention has a well-established utility, *i.e.*, a utility that will be immediately appreciated by one of ordinary skill in the art based on the characteristics of the invention, regardless of whether any such utility has been asserted. Neither should any rejection be made for lack of utility if an applicant has asserted a specific and substantial utility that would be considered credible by one of ordinary skill in the art.

In most cases, an applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement of 35 U.S.C. §101. MPEP §2107.02 III A. The Court of Customs and Patent Appeals stated in *In re Langer*:

> As a matter of Patent Office practice, a specification which contains a disclosure of utility which corresponds in scope to the subject matter sought to be patented must be taken as sufficient to satisfy the utility requirement of §101 for the entire claimed subject matter unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope.

In re Langer, 503 F.2d 1380, 1391, 183 USPQ 288, 297 (CCPA, 1974, emphasis in original). To overcome the presumption of sufficient utility as asserted by an applicant, the Examiner must carry the initial burden to make a *prima facie* showing of lack of utility and provide a sufficient evidentiary basis for the conclusion. In other words, the Examiner "must do more than merely question operability--[he] must set forth factual reasons which would lead one skilled in the art to question objective truth of the statement of operability." *In re Gaubert*, 524 F.2d 1222, 1224, 187 USPQ 664, 666 (CCPA 1975).

As MPEP §2107.02 IV further states, a detailed explanation should be given for a utility rejection as to why the claimed invention has no specific and substantial asserted utility. Documentary evidence should be provided when possible. Otherwise the Examiner should specifically explain the scientific basis for his factual conclusions.

2. Rejection of claims 3, 6, 7, 13, 30, and 31

Claims 3, 6, 7, 13, 30, and 31 were rejected under 35 U.S.C. § 101 as allegedly lacking utility. Appellant respectfully traverses this rejection and argues that the rejection is improper. The present invention resides, in part, in the identification of TGR18 nucleic acids. The invention satisfies utility under 35 U.S.C. § 101 because the identification of TGR18 nucleic acids permits one of skill in the art to analyze modulators of TGR18. Such modulators can be used, *e.g.*, for altering TGR18 function in the kidney and for the treatment of kidney-related conditions such as hypertension.

In the Final Office Action mailed February 20, 2004, the Examiner concedes that the invention has a credible utility (section 12, page 14), but alleges that insufficient evidence of a specific and substantial utility has been provided by Appellant. The Examiner argues that Appellant has not established anything specific about the receptor such as its tissue of origin or "the properties of the claimed GPCR" (section 10, page 3 of the Final Office Action). Furthermore, he contends that the Declaration under 37 C.F.R. § 1.132 by Daniel Lin filed October 15, 2003 ("Lin Declaration I", Exhibit 1 of the Evidence Appendix) to provide additional evidence of the utility of the claimed invention merely shows that a polypeptide having a sequence of SEQ ID NO:2, which is encoded by SEQ ID NO:1, can increase intracellular calcium. The Examiner concludes that this showing does not establish a specific utility because many GPCRs modulate changes in intracellular calcium when generally stimulated (section 10, page 3 of the Final Office Action). The Examiner also argues that the asserted utility is not substantial, as further research is required to determine how to use the claimed receptor.

In the Advisory Action mailed September 7, 2004, the rejection for lack of utility is maintained. The Examiner alleges that a second Declaration under 37 C.F.R. § 1.132 by Daniel Lin filed August 11, 2004 ("Lin Declaration II", Exhibit 2 of the Evidence Appendix) was taken into consideration, but again fails to provide sufficient evidence of specific and substantial utility.

3. The asserted utility is specific, substantial, and credible

As described in the present application, the present inventors cloned, for the first time, the mouse polynucleotide sequence encoding a GPCR referred to in the specification as "TGR18". The inventors also identified the amino acid sequence of mouse TGR18, and determined the tissue-specific expression pattern of TGR18 mRNA (*e.g.*, pages 55, 58 and 59).

The specification further teaches that mouse TGR18 is a GPCR (*see, e.g.*, page 10, lines 11-19); that mouse TGR18 is abundantly expressed in the kidney (*see, e.g.*, page 7, lines 7-9); and that TGR18 can participate in the modulation of cellular function in cells, for example kidney cells, in which it is expressed (*see, e.g.*, page 51, lines 31-34). The specification also discloses that a GPCR that is predominantly expressed in the kidney can play a role in renal disease, *e.g.*, hypertension (*see, e.g.*, page 52, lines 2-6) and in modulating kidney cell function (*see, e.g.*, page 7, lines 7-8 and 12-14). Appellant also teaches that TGR18 can be use to identify modulators of GPCR activity and that GPCR activity can be assessed using a variety of assays to determine functional effects (*see, e.g.*, the section beginning on page 29). These include changes in calcium ion levels (*see, e.g.*, page 11, lines 8-10 and page 39, lines 27-30).

As further evidence that TGR18 has GPCR activity, Appellant provided Lin Declaration I. The Declaration presents data demonstrating that murine TGR18, which is encoded by SEQ ID NO:1, has a known G-protein coupled receptor activity, *i.e.*, it transduces an increase in intracellular calcium. As Dr. Lin explains, GPCR activity can be assessed using a variety of common assays. One such assay is an Aequorin assay, which is widely used in the art to measure GPCR-mediated increases in intracellular calcium. The data provided in the Lin Declaration I establish that mouse (encoded by the coding region of SEQ ID NO:1), human, and rat TGR18 GPCR all transduce an increase in intracellular calcium.

As explained in the section summarizing the rejections, the Examiner argued that Lin Declaration I was insufficient to establish a specific utility because many GPCRs can transduce an increase in cellular calcium, and there was no evidence that succinic acid (which was the ligand in the experiments summarized in the Lin Declaration I; the ligand was not disclosed in the specification) was biologically significant in the kidney and did not serve merely as a general

stimulator of GPCR activity. Although Appellant believes that the Lin Declaration I, which provided data showing that mouse TGR18 is a functional GPCR, is more than sufficient to support the asserted utilities, further evidence, *i.e.*, a post-filing publication, that TGR18 has biological activity in kidney function was provided in Appellant's response mailed on August 11, 2004.

The publication (He *et. al., Nature*, 429:188-193, 2004), a copy of which is provided in Exhibit 3 of the Evidence Appendix of this brief, describes that mouse GPR91 (which corresponds to TGR18; *see,* the Lin Declaration II accompanying the August 11, 2004 response) has GPCR activity. He *et al.* also teach that the role of succinate as a ligand for a GPCR was an unexpected finding (*see, e.g.,* the Abstract, page 188, column 1). Accordingly, succinate is not regarded by those in the art as a general stimulator of GPCR activity. Thus, the Examiner's speculations that succinate may be a general stimulator of GPCR activity, which were presented in response to Lin Declaration I, are untrue. TGR18 is, in fact, specifically activated and therefore has a specific function.

He *et al.* also describe earlier work in the field that provides a biologically relevant context for succinate and consequently, TGR18, in the kidney. For example, succinate was known to increase the re-absorption of phosphate and glucose into the proximal tubule (*see,* page 192, the first full paragraph of column 1). Further, succinate-treated kidney culture *ex vivo* release renin (page 191, first column, last paragraph). These effects were known prior to the filing date of the application, as evidenced by the publication dates of the references cited at the indicated passages.

Moreover, He *et al.* provide data showing that succinate induces hypertension in normal mice. In GPR91-deficient animals (*i.e.,* TGR18-deficient animals), however, succinate did not induce hypertension (*see, e.g.,* page 191, second column, last paragraph). Thus, He *et al.* provide additional data demonstrating the biological relevance of TGR18 in the kidney, as taught in the instant application. In view of the biological role of TGR18 and its demonstrated GPCR function, TGR18 nucleic acid and polypeptides sequences have credible, substantial, and specific uses.

Specific utility

Specific utility is defined by the MPEP as a utility that is specific to the subject matter claimed. The MPEP explains that applications show sufficient specific utility when applicants disclose a "specific biological activity" and reasonably correlate that activity to a "disease condition." MPEP §§2107.01 and 2107.02. In the present application, Appellant identifies the nucleic acid and amino acid sequences of mouse TGR18, demonstrates the expression pattern of TGR18, and discloses that TGR18 plays a role a "disease condition" (*e.g.*, hypertension (page 52, lines 2-6) that correlates with a "biological activity" (*i.e.*, GPCR activity). The application provides methods for identifying compounds capable of modulating TGR18 activity and therefore for modulating TGR18 in a biological context *in vivo*, *e.g.*, in the kidney. Appellant thus submits that the present invention has a utility that is specific for the claimed TGR18 GPCRs.

Substantial utility

The Examiner's position that the asserted utility is not substantial because more research is required to determine how to use the claimed (section 10, page 3 and bridging to page 4 of the Final Office Action) is also unfounded.

A substantial utility defines a real world use (MPEP§2107.01.I). This invention provides TGR18 polynucleotide and polypeptide sequences. As explained above, the application teaches that TGR18 is expressed in the kidney and that kidney-specific GPCRs can function in diseases such as hypertension. The application further teaches how to identify agonists and antagonists of TGR18. Appellant has provided additional evidence as to the biological significance of TGR18 *in vivo*. The present invention therefore has a real-world use in modulating the physiology of tissues, *e.g.*, kidney, in which TGR18 is expressed. Accordingly, the utilities satisfy the "substantial" criterion for utility.

4. Finding Sufficient Utility in the Present Application is Consistent with the Policy of Encouraging Early Disclosure

Our patent law places much emphasis on encouraging early disclosure of inventions. This is a particularly relevant policy consideration in case law involving the utility standard under 35 U.S.C. §101. In *Brenner v. Manson*, 383 U.S. 519, 148 USPQ 689 (US Sup. Ct. 1966), for instance, the Supreme Court ruled that a process to produce a compound may be patented only if the compound has "substantial utility," "specific benefit ... in currently available form." Whether granting patent protection to the discovery of a new process or compound with a yet unknown practical utility would encourage prompt disclosure of inventions was one factor the Court carefully considered and to a significant extent relied upon in reaching its landmark decision. 383 U.S. at 534-35, 148 USPQ at 695.

In *Nelson v. Bowler*, 626 F.2d 853, 206 USPQ 881 (CCPA 1980), the CCPA was confronted with a situation where the claimed compound, 16-phenoxy-substituted prostaglandin (PG), was shown to have some pharmacological activity, *i.e.*, causing changes in blood pressure in the rat blood pressure (BP) test and stimulation of smooth muscles in the gerbil colon smooth muscle stimulation (GC-SMS) test, yet no specific therapeutic use for the compound was established. In deciding the question of utility, the CCPA stated:

> Knowledge of the pharmacological activity of any compound is obviously beneficial to the public. It is inherently faster and easier to combat illness and alleviate symptoms when the medical profession is armed with an arsenal of chemicals having known pharmacological activities. Since it is crucial to provide researchers with an incentive to disclose pharmacological activities in as many as compounds as possible, we conclude that adequate proof of any such activity constitute a showing of practical utility.

Nelson, 626 F.2d at 856, 206 USPQ at 883. The present case is analogous to *Nelson*. TGR18 has a physiological functions in the kidney and compounds capable of modulating TGR18 are useful as agents for regulating its function. Assays for screening for GPCR modulators and evaluating their function, including in model systems such as mouse model systems, are thus beneficial to the public and the disclosure of how to perform these assays should be encouraged. The present application provides just this kind of disclosure. A holding that the present invention lacks sufficient utility

under 35 U.S.C. §101 to warrant patent protection would be inconsistent with the underlying policy of case law and create a strong disincentive for researchers to disclose their inventions of this type.

5. Summary

In light of the foregoing discussion, Appellant has shown that TGR18 nucleic acid and polypeptide sequences have credible, specific, and substantial utility. The utility rejection under 35 U.S.C. §101 is therefore improper and should be reversed.

B. Rejections under 35 U.S.C. § 112--enablement

1. Rejection of claims 3, 6, 7, 13, 30, and 31

Claims 3, 6, 7, 13, 30 and 31 were rejected as not being enabled, on the ground that the claimed invention is not supported by a specific and substantial asserted utility or a well-established utility. As discussed above, the claimed invention has a credible, specific and substantial utility under the examination guideline set forth in the MPEP. Appellant therefore believes that the utility-based enablement rejection under 35 U.S.C. §112, first paragraph, is improper and should be withdrawn.

2. Rejection of claims 3, 6, 7, 13, 30, and 31--relating to variants and fragments

Claims regarding variants and fragments of SEQ ID NO:1 are additionally rejected under 35 U.S.C. § 112, first paragraph for alleged lack of enablement. This appears to at least minimally relate to claims 3 and 13, although this was not articulated by the Examiner. The rejection is therefore discussed below as it relates to each of claims 3, 6, 7, 13, 30 and 13.

i. Standards for enablement

It is well-settled in the biotechnology art that routine screening of even large numbers of samples is not undue experimentation when a probability of success exists. *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988). As stated in *Wands*, "enablement is not

precluded by the necessity for some experimentation, such as routine screening." *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404 (Fed. Cir. 1988). Moreover, as set forth in MPEP § 2164.08, a rejection for undue breadth is inappropriate where a nucleic acid encodes a particular protein sequence and "one of skill could readily determine any one of the claimed embodiments."

In order to establish a *prima facie* case of enablement, the examiner has the initial burden to establish a reasonable basis to question the enablement provided for the claimed invention (*In re Wright*, 999F.2d 1557, 1561-62, 27 USPQ2d 1510, 1513 (Fed. Circ. 1993).

ii. Examiner's arguments

In the Final Office Action mailed February 20, 2004, the Examiner alleges that the specification lacks adequate guidance regarding the structural features that are required for activity and that it would require undue experimentation to make and use the claimed nucleic acid sequences. In particular, the Examiner argues that it would require undue experimentation to identify the claimed sequences because of alleged difficulties in predicting structure/function relationships. In section 20 of the Final Office Action, the Examiner contends that at a minimum, a GPCR would have 230 residues and concludes that it would therefore be physically impossible to have a GPCR with only 200 amino acids. In section 21, the Examiner further argues that Appellant has provided little or no guidance in terms of identifying positions in the TGR18 protein that are tolerant to change.

The Examiner responded to Appellant's response filed October 15, 2003, by further maintaining that insufficient guidance is presented to support the undertaking of screening, isolating, and characterizing all the fragments and sequences derivatives currently claimed. The Examiner takes the position that the guidance in the specification merely suggests possible results (section 25 of the Final Office Action), that no concrete structural or functional parameters are present (section 26) and that no embodiments have been enabled (section 27). These arguments contend that no success can be expected as extensive research is required to first characterize SEQ ID NO:1 before undertaking experimentation to screen, isolate, and characterize all of the fragments and sequence variants claimed.

Appellant respectfully traverses. As detailed below, the application provides guidance to make the claimed sequences based on structural properties provided in the specification and known in the art, and guidance for performing assays to assess the function of the sequences. Thus, although such analyses could conceivably require analyzing a large number of sequences, the practitioner could reasonably expect to be able to successfully identify sequences that fall within the scope of the invention. Further, the Examiner fails to establish a *prima facie* case that the claims are not enabled because the evidence provided in support of the arguments fails to support those arguments.

iii. Claims 3, 6, 7, 13, 30, and 31 are enabled

In the present case, the claims are drawn to isolated TGR18 nucleic acids that encode a polypeptide that has a G protein coupled receptor activity, *i.e.,* the ability to transduce an increase in intracellular calcium, and structural features set forth in the claims, *i.e.,* reference nucleic acid or amino acid sequences. As noted by Appellant in the response filed on October 15, 2003, the application teaches reference nucleic acid and polypeptide sequences (SEQ ID NOs: 1 and 2, respectively), and describes the known structural features of GPCRs (*see, e.g.,* page 10, lines 20-27). Further, related GPCR polypeptide sequences, *e.g.,* human sequences, have been disclosed (*see, e.g.,* U.S. Patent No. 5,871,963, submitted in Appellant's Information Disclosure Statement dated October 9, 2001; copy provided in Exhibit 4 of the Evidence Appendix). The practitioner is also directed to routine techniques for making TGR18 nucleic acid sequences (*see, e.g.,* the section starting on page 24 of the application), and commonly used methods to assess GPCR activity, including methods of determining changes in intracellular calcium ion levels (*see, e.g.,* page 41, line 30, bridging to page 42, line 8 and page 43, lines 3-10). Thus, the disclosure in the specification, along with methodology well known to those of skill in the art, provide ample direction for screening nucleic acids encoding GPCRs having the claimed structural and functional characteristics. The rejection will be discussed below with regard to each claim.

Claim 3 (and claims 30 and 31 as they relate to claim 3)

Regarding the issue of enablement of nucleic acids where a large number of possible embodiments exist, the PTO has provided express guidelines for examination. As noted above, a rejection for undue breadth is inappropriate where one of skill could readily determine any one of the claimed embodiments (MPEP § 2168.08). In the present application, one of skill needs to identify nucleic acids that have a high level of identity with respect to a conserved reference sequence (*see, e.g.,* claim 13, which is addressed separately below), or that have a particular number of contiguous amino acids with reference to a conserved reference sequence, as recited in claim 3. Although many such nucleic acids are possible, one of skill can readily determine, one by one, any particular sequence that has these properties without undue experimentation.

Claim 3 relates to 1 nucleic acid encoding a polypeptide comprising 200 amino acids of SEQ ID NO:2. The Examiner first argues that it would be physically impossible to have a GPCR with only 200 amino acids. Appellant notes that the specification teaches chimeric molecules (*see, e.g.,* page 40, lines 11-25) in which domains or other regions of the claimed sequences are used in the context of a heterologous protein that have activity. Thus, it is in fact possible to have a polypeptide that has GPCR activity and comprises only 200 amino acids of the claimed sequence.

Appellant further asserts that one of skill could reasonably expect to identify fragments comprising 200 amino acids of the reference sequence that can be expected to be active. As the Examiner acknowledges in section 20 of the Final Office Action, much is known about the structure of GPCR domains. Furthermore, Appellant has shown that TGR18 has GPCR activity, *e.g.,* it transduces an increase in intracellular calcium, and provided guidance in performing such assays (as noted above). The TGR18 amino acid sequence shown in SEQ ID NO:2 is 317 amino acids. The nucleic acids of claim 3 encode a sequence that comprises at least 200 amino acids in length, *i.e.,* a substantial portion of the sequence. The Examiner fails to provide proper evidence that one of skill could not reasonably expect to use the detailed knowledge of the structure of GPCR domains and select fragments of SEQ ID NO:2 that would have activity, or be useful for identifying agents, *e.g.,* antibodies, that bind to TGR18 (*see, e.g.,* the specification page 32, lines 22-27). The references cited by the Examiner (section 21, page 7 of the Final Office Action) only teach that it

can be difficult to determine whether a potential gene discovered solely by evaluating sequence is truly functional. Here, the facts are different. TGR18 possesses GPCR activity and has a biological function. The references cited by the Examiner are therefore not applicable to the case at hand.

Further, the references cited in section 21, page 6 of the Final Office Action merely teach that it is possible to mutate proteins and alter function. For example, Wells (*Biochemistry* 29:8509-8517, 1995) teaches only that designing large changes in function will often require mutation of more than one functional residue (*see, e.g,* the first paragraph of page 8509). These references collectively support Appellant's position that identifying nucleic acids encoding TGR18 polypeptides comprising at least 200 amino acids of SEQ ID NO:2 is in fact routinely performed in the art. Thus, the Examiner has failed to meet his burden in establishing that the claims are not enabled.

Claim 6 (and claims 30 and 31 as they relate to claim 6)

Insofar as the enablement rejection regarding variants, muteins and fragments of the TGR18 sequences of the invention applies to claim 6, Appellant traverses. As explained in the section of this brief relating to utility, TGR18 functions as a GPCR and has a biological role. Thus, SEQ ID NO:2 does not require further characterization. The MPEP is clear

> [W]hen claims are directed to any purified and isolated DNA sequence encoding a specifically named protein where the protein has a specifically identified sequence, a rejection of the claims as broader than the enabling disclosure is generally not appropriate because one skill in the art could readily determine any one of the claimed embodiments. (MPEP § 2164.08)

This is precisely the case here. Accordingly, the rejection as it relates to claim 6 is improper.

Claim 7 (and claims 30 and 31 as they relate to claim 7)

Insofar as the rejection relates to claim 7, Appellant traverses. Claim 7 recites a specific sequence, SEQ ID NO:1. SEQ ID NO:1 encodes a polypeptide that has GPCR activity. Thus, the Examiner's position that one of skill could not identify nucleic acid variants that encode functional TGR18 polypeptides is not applicable here.

Claim 13 (and claims 30 and 31 as they relate to claim 3)

Claim 13 encompasses nucleic acids encoding TGR18 polypeptides comprising 95% or greater amino acid identity to the amino acid sequence of SEQ ID NO:2. As explained above with regard to claim 3, the Examiner fails to provide proper evidence that one of skill could not reasonably expect to use the detailed knowledge of the structure of GPCR domains and select variants that would have GPCR activity. In addition to the guidance provided in the specification for performing TGR18 functional assays, sequence alignment algorithms are well-known in the art (*see, e.g.,* the direction provided in the specification at the passage starting at page 18, line 32). The references cited by the Examiner in section 21 of the Final Office Action provide no evidence that one of skill could not reasonably determine residues to be substituted or mutated such that an active sequence that is almost identical, *i.e.,* is at least 95% identical, could be generated based on a comparison between the mouse and other known GPCR sequences. As noted above, a related human polypeptide sequence was disclosed in U.S. Patent No. 5,871,963. Indeed, He *et al., supra,* also show that human and rat GPR91 (*i.e.,* human and rat TGR18) are active, thus providing additional evidence that variant TGR18 sequences having GPCR activity can be readily identified based on sequence comparison and alignment.

iv. Summary

In light of the foregoing discussion, Appellant has provided ample guidance for one of skill to make and use the claimed nucleic acid sequences. Accordingly, the claims are enabled. Appellant therefore requests that that the enablement rejection under 35 U.S.C. §112 be reversed.

C. Rejection under 35 U.S.C. § 112, written description

1. Standards for written description

Written description does not required that the disclosure as originally filed provide *in haec verba* support for claimed subject matter (*see, e.g., Purdue Pharma L.P. V. Faulding, Inc.* 230 F.3d 1320, 1323, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000)). It is not necessary that the application

describe the claim limitations exactly, but only to the extent that one of ordinary skill in the art would recognize from the disclosure that appellants invented the subject matter (*In re Herschler*, 591 F.2d 693, 200 USPQ 711 (CCPA 1979).

The Federal Circuit has expressly considered the application of the written description requirement to inventions in the field of biotechnology. See, *University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997). The Court held that the written description requirement can be fulfilled in any number of ways, so long as the specification describes the invention in sufficient detail that one skilled in the art can clearly conclude that the inventor invented the claimed invention. For a chemical invention, an adequate description "requires a precise definition, such as by structure, formula, chemical name, or physical properties...." (119 F.3d at 1568, 43 USPQ2d at1406.) The Court also addressed the manner by which a genus of cDNAs might be described, when it stated:

> A description of a genus of cDNAs may be achieved by means of a recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to the members of the genus, which features constitute a substantial portion of the genus. (also 119 F.3d at 1568, 43 USPQ2d at 1406).

Written description requirement in the context of DNA-related inventions has been further discussed by the Federal Circuit in *Enzo Biochem. Inc. v. Gen-Probe Inc.*, 296 F.3d 1316, 63 USPQ2d 1609 (Fed. Cir. 2002). In *Enzo*, the Court defined a standard where "the written description requirement can be met by 'showing that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics... i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics." F.3d 1316 at 1324, 63 USPQ2d at 1613.

2. Examiner's arguments

The Examiner argues that with the exception of SEQ ID NO:1 and SEQ ID NO:2, the skilled artisan cannot envision the detailed structure of the polypeptides encompassed by the

claims because variant sequences were not specifically set out in the specification (see, section 13 of the Office Action mailed April 15, 2003). In the Final Office Action mailed February 10, 2004, the Examiner also argues that Appellant is claiming sequence derivations of an invention that is without utility and that is not enabled, and then concludes that *University of California v. Eli Lilly, & Co., supra* is not applicable here (sections 30-33). In particular, the Examiner argues that percent identity is an insufficient structural parameter and has no functional value (section 32); and that there is no assay that can be used to validate the identity of a TGR18 GPCR (section 33).

The Examiner further contends that the fact pattern in the instant application does not correspond to Example 14 (cited in Appellant's response mailed for filing on October 15, 2003), which recites percent identity, of the "Revised Interim Written Description Guidelines", Federal Register, Vol. 66, No. 4, 1099, January 5, 2001, because the enzymes in Example 14 are not analogous to GPCR polypeptides (section 33 of the Office Action). Appellant respectfully traverses this rejection.

3. Claims 3, 6, 7, 13, 30, and 31 are adequately described

In the present case, the claims are drawn to isolated TGR18 nucleic acids that encode a polypeptide that has a G protein coupled receptor activity. Structural features are set forth in the claims, *i.e.,* reference nucleic acid or amino acid sequences. Thus, the claim language defines physical and structural properties of the invention, as explicitly required by the Court in *University of California v. Eli Lilly & Co.* Furthermore, the polypeptides have a function, *e.g.,* the ability to transduce an increase in intracellular calcium. Contrary to the Examiner's assertion that Appellant has not described a useful invention, the claimed invention does, in fact, meet the requirement for utility, as demonstrated above. The Examiner's argument that the facts here are different from the *University of California v. Eli Lilly & Co.* and that no useful or enabled TGR18 nucleic acids are described is therefore totally unfounded.

Appellant notes that aspects of the written description rejection appear to relate more to enablement, thus, the explanations below they will also be addressed to some extent, in terms of

the "description" of the guidance in the specification. The rejection will also be discussed as it relates to each claims.

Claim 3 (and claims 30 and 31 as they relate to claim 3)

Claim 3 is drawn to nucleic acids encoding polypeptides comprising at least 200 amino acids of SEQ ID NO:2. Such a polypeptide comprises a substantial portion of SEQ ID NO:2. Clearly, this claim sets forth structural properties defining the genus of nucleic acids.

Further, as repeatedly pointed out above, the application teaches TGR18 functional properties, *i.e.,* GPCR activity, such as transducing increases in intracellular calcium. GPCRs generally have conserved structural features, as noted by the Examiner in section 35 of the Office Action, and as taught in the specification on page 10. In addition, exemplary conservative substitutions are described in the section beginning on page 15 of the application. Furthermore, again as explained above, related sequences, *e.g.,* the human polypeptide sequence disclosed in U.S. Patent No. 5,871,963, are known. Thus, the claimed GPCR sequences of the instant application do not exist in a context devoid of the knowledge of similar sequences. In view of this level of knowledge and Appellant's demonstration of identifying functional and structural characteristics of the claimed genus, the claims are properly described under the standards articulated by the Court in *University of California v. Eli Lilly & Co. Enzo Biochem. Inc. v. Gen-Probe Inc.*, both *supra.* It is therefore respectfully requested that this rejection be reversed.

Claim 6 (and claims 30 and 31 as they relate to claim 6)

Claim 6 recites an isolated nucleic acid encoding a polypeptide comprising an amino acid sequence of SEQ ID NO:2. It has been established by the courts that, since the genetic code is widely known, a disclosure of an amino acid sequence provides sufficient information such that one would accept that an applicant was in possession of the full genus of nucleic acids encoding a give amino acid sequence (*see, e.g., In re Bell*, 991 F.2d 781, 26 USPQ2d 1529 (Fed. Cir. 1993). Accordingly claim 6 meets the written description requirement. Appellant therefore requests that the rejection be overturned.

Claim 7 (and claims 30 and 31 as they relate to claim 7)

Claim 7 recites an isolated nucleic acid that comprises SEQ ID NO:1. SEQ ID NO:1 clearly provides a precise structural feature of the claimed nucleic acids. Appellant therefore requests reversal of this rejection.

Claim 13 (and claims 30 and 31 as they relate to claim 13)

The Examiner appears to be arguing in section 32 of the Final Office Action that percent identity, as cited in claim 13, is an insufficient structural parameter with no functional value, particularly in view of the alleged lack of utility of the reference sequence. He further contends that the fact pattern in the instant application does not correspond to Example 14, which recites percent identity, of the "Revised Interim Written Description Guidelines" (the "Revised Guidelines"), Federal Register, Vol. 66, No. 4, 1099, January 5, 2001, because the enzymes in Example 14 are not analogous to GPCR polypeptides (section 33 of the Office Action). Appellant disagrees.

The examples in the Revised Guidelines directly relate to the present claims. In Example 14 of the Guidelines, variants of a protein that have at least 95% identity to a sequence and catalyze a reaction, *i.e.*, they have a function, are claimed. Example 14 describes, but does not exemplify, variants of the protein, including substitutions, deletions, and insertions, and indicates that procedures for making the protein are routine in the art. The specification also describes an assay for detecting protein activity. The analysis indicates that the single disclosed species is representative of the claimed genus because all members must have the particular structural feature, at least 95% identity to the reference compound, and functional feature. The Conclusion of Example 14 states that the disclosure meets the requirements of 35 U.S.C. § 112 as providing adequate written description for the claimed invention.

As noted above, the TGR18 sequences of the instant application, as with the sequence in Example 14 of the Revised Guidelines, do not exist in a context devoid of the knowledge of similar sequences. GPCRs that have conserved structural features are known; GPCR

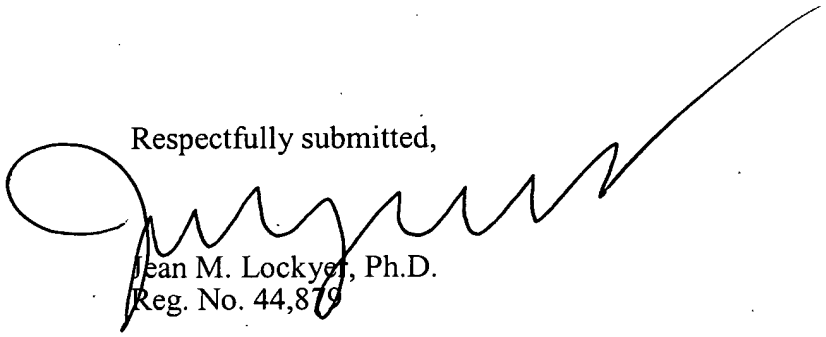
assays are taught in Appellant's specification and known in the art; and exemplary conservative substitutions are taught in the specification. Thus, the facts here comport with Example 14 of the Guidelines.

Further, as noted above, claim 13, recites both structural and functional elements that are common to the genus. Accordingly, the claims meet the written description requirement. Appellant therefore respectfully requests that the rejection be reversed.

D. Conclusion

Appellant has addressed each of the utility, enablement, and written description rejections as it applies to the pending claims. In each instance, Appellant has met the legal standards. Appellant therefore believes all claims now pending in this Application are in condition for allowance.

Respectfully submitted,



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60353968 v1

VIII. CLAIMS APPENDIX

Claims involved in the appeal:

3. (previously presented) An isolated nucleic acid encoding a polypeptide comprising at least 200 contiguous amino acids of SEQ ID NO:2.

6. (previously presented) An isolated nucleic acid encoding a polypeptide comprising an amino acid sequence of SEQ ID NO:2.

7. (previously presented) The isolated nucleic acid of claim 6, wherein the nucleic acid comprises the nucleotide sequence of SEQ ID NO:1.

13. (previously presented) An isolated nucleic acid encoding a G-protein coupled receptor polypeptide that transduces an increase in intracellular calcium, wherein the nucleic acid encodes a polypeptide comprising 95% or greater amino acid identity to the amino acid sequence of SEQ ID NO:2.

30. (previously presented) An expression vector comprising the nucleic acid of any one of claims 3, 6, 7, or 13.

31. (original) A host cell transfected with the vector of claim 30.

IX. EVIDENCE APPENDIX

1. Declaration Under 37 CFR 1.132 by Daniel Lin, Ph.D. (Lin Declaration I)

    a) filed with Appellant's amendment dated October 15, 2003

    b) entry of the amendment was acknowledged in the Office Action dated February 20, 2004

2. Declaration Under 37 CFR 1.132 by Daniel Lin, Ph.D. (Lin Declaration II)

    a) filed on August 11, 2004 with Appellant's amendment after final rejection

    b) The Advisory Action mailed September 7, 2004 stated that the amendment and response filed August 11, 2004 was entered in full and the Declaration was taken into consideration.

3. Weihal He et al., "Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors," Nature (13 May 2004) 429: 188-193

    a) submitted on August 11, 2004 with Appellant's amendment after final rejection

    b) The Advisory Action mailed September 7, 2004 stated that the amendment and response filed August 11, 2004 was entered in full and the Declaration was taken into consideration.

4. U.S. Patent No. 5,871,963

    a) submitted in Appellant's IDS dated October 9, 2001

    b) initialed by Examiner April 8, 2003

    c)) cited in Appellant's amendment filed August 11, 2004 after final rejection

    d) The Advisory Action mailed September 7, 2004 stated that the amendment and response filed August 11, 2004 was entered in full and the Declaration was taken into consideration.

PATENT
Attorney Docket No.: 018781-006210US
Client Ref. No.: T00-013-1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

LIN et al.

Application No.: 09/891,138

Filed: June 25, 2001

For: NOVEL RECEPTORS

Customer No.: 20350

Confirmation No.: 8826

Examiner: Christopher J. Nichols

Art Unit: 1647

DECLARATION UNDER 37 C.F.R. 1.132 BY DANIEL LIN, PH.D.

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I, Daniel Lin, Ph.D., am a scientist at Tularik Inc., a biopharmaceutical company headquartered in South San Francisco, CA and the assignee of the above-referenced patent application. I am the inventor of the subject matter disclosed and claimed in the above-referenced patent application.

2. I hold a Ph.D. from the Massachusetts Institute of Technology (1999). I have worked in the field of G protein-coupled receptors for about four years.

3. It is my understanding that the claims currently under examination, which relate to, *inter alia*, murine TGR18 nucleic acid sequences, were rejected as allegedly lacking utility. This

Declaration presents exemplary data showing that TGR18 functions as a GPCR. The experiments were performed by myself or under my supervision.

4. GPCR activity can be assessed using a variety of common assays. One such assay is an Aequorin assay. Aequorin assays are widely used in the art to measure GPCR-mediated increases in intracellular calcium. The assay involves the use of the Ca^{2+}-sensitive photoprotein aequorin. The aequorin complex contains the apo-aequorin protein, molecular oxygen, and the luminophore coelenterazine. The binding of calcium ions disrupts the complex, leading to the emission of blue light, which provides a means of determining increases in intracellular calcium.

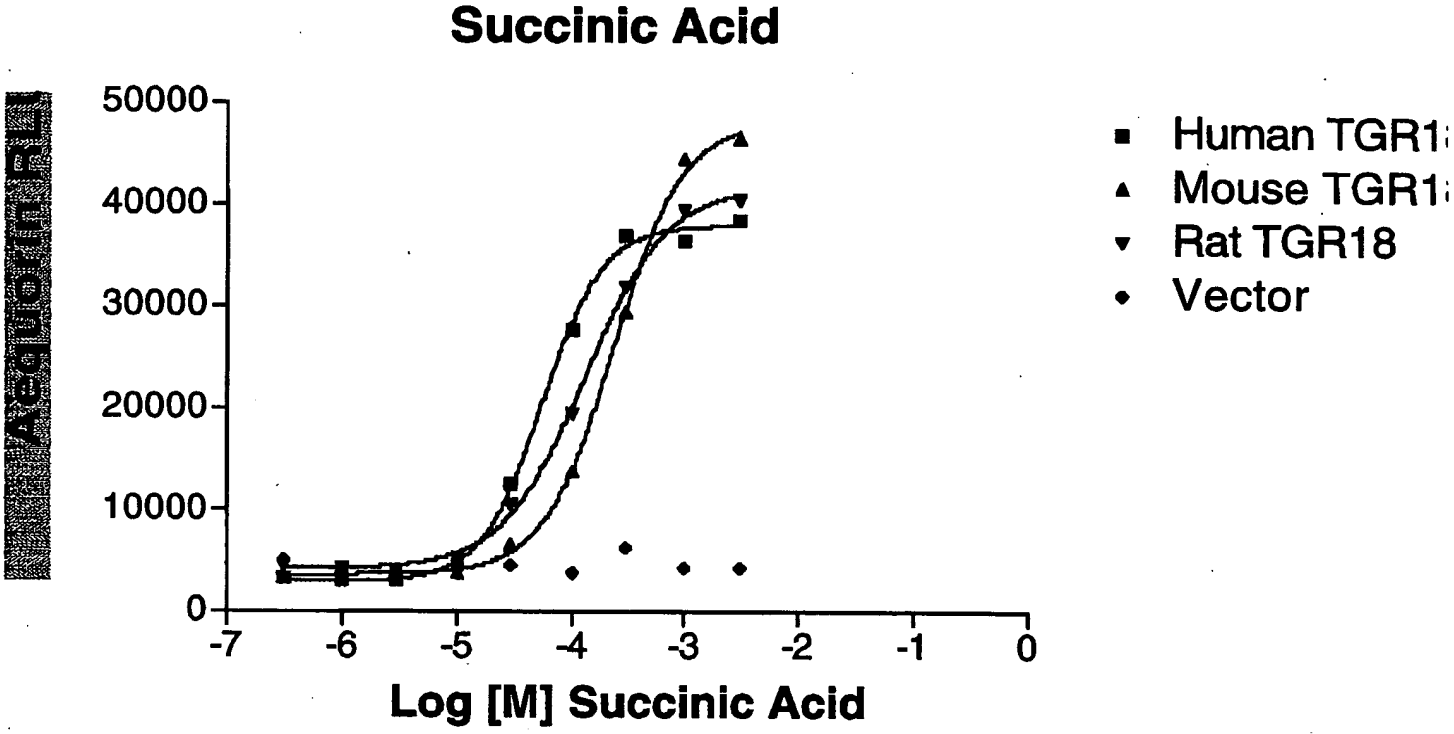
5. Mouse, human, and rat TGR18 GPCR activities were tested in an Aequorin assay. Briefly, CHO cells were transiently co-transfected with 10 μg of an Aequorin reporter gene and 10 μg of a cDNA encoding human TGR18, mouse TGR18, rat TGR18, or a vector control. The mouse TGR18 expression vector comprises the coding region of the cDNA sequence presented in SEQ ID NO:1, which encodes the protein of SEQ ID NO:2. Following transfection, the cells were harvested and re-suspended in buffer containing coelenterazine f. Aequorin luminescence was determined following incubation of the harvested cells with ligand. The results, shown in Figure 1, demonstrate that mouse, human, and rat TGR18 all have GPCR activity: they each transduce an increase in intracellular calcium.

6. I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both (18 U.S.C./1001), and may jeopardize the validity of the patent application or any patent issuing thereon.

Date: Oct 15, 2003 By: _____

Daniel Lin, Ph.D.

Figure 1


Succinic Acid
Aequorin RLU
50000
40000
30000
20000
10000
0
-7
-6
-5
-4
-3
-2
-1
0
Log [M] Succinic Acid
Human TGR1
Mouse TGR1
Rat TGR18
Vector

PATENT
Attorney Docket No.: 018781-006210US
Client Ref. No.: T00-013-1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

LIN et al.

Application No.: 09/891,138

Filed: June 25, 2001

For: NOVEL RECEPTORS

Customer No.: 20350

Confirmation No.: 8826

Examiner: Christopher J. Nichols

Art Unit: 1647

DECLARATION UNDER 37 C.F.R. §1.132 BY DANIEL LIN, PH.D.

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. I, Daniel Lin, Ph.D., am a scientist at Tularik Inc., a biopharmaceutical company headquartered in South San Francisco, CA and the assignee of the above-referenced patent application. I am the inventor of the subject matter disclosed and claimed in the above-referenced patent application. I am also an author on the article by He, *et. al.*, in *Nature* 429:188-193, 2004, a copy of which accompanies the response submitted with this Declaration.

2. The mouse G-protein-coupled receptor GPR91 that is a subject of the *Nature* article is the same as mouse TGR18 of the current application, *i.e.*, it is encoded by the coding region of the nucleic acid sequence shown as SEQ ID NO:1 on page 58 of the specification. This nucleic acid encodes the polypeptide shown in SEQ ID NO:2. All of the experiments relating to mouse

GPR91 in the *Nature* article employed this sequence, unless otherwise specifically indicated in the paper.

3. I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon.

Date: 7/26/2004 By: _____

Daniel Lin, Ph.D.

Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors

Weihai He[1]*, Frederick J.-P. Miao[1], Daniel C.-H. Lin[1], Ralf T. Schwandner[1,2], Zhulun Wang[1], Jinhai Gao[1]*, Jin-Long Chen[1], Hui Tian[1] & Lei Ling[1]

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[2]*Tularik GmbH, Josef-Engert-Strasse 9, 93053 Regensburg, Germany*

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The citric acid cycle is central to the regulation of energy homeostasis and cell metabolism[1]. Mutations in enzymes that catalyse steps in the citric acid cycle result in human diseases with various clinical presentations[2]. The intermediates of the citric acid cycle are present at micromolar concentration in blood and are regulated by respiration, metabolism and renal reabsorption/extrusion. Here we show that GPR91 (ref. 3), a previously orphan G-protein-coupled receptor (GPCR), functions as a receptor for the citric acid cycle intermediate succinate. We also report that GPR99 (ref. 4), a close relative of GPR91, responds to α-ketoglutarate, another intermediate in the citric acid cycle. Thus by acting as ligands for GPCRs, succinate and α-ketoglutarate are found to have unexpected signalling functions beyond their traditional roles. Furthermore, we show that succinate increases blood pressure in animals. The succinate-induced hypertensive effect involves the renin–angiotensin system and is abolished in GPR91-deficient mice. Our results indicate a possible role for GPR91 in renovascular hypertension, a disease closely linked to atherosclerosis, diabetes and renal failure[5,6].

In a search for natural ligands for orphan GPCRs, we tested extracts from various animal tissues for their ability to evoke an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) using the aequorin assay[7]. We found that fractions from pig kidney extracts specifically activated cells expressing GPR91 (Fig. 1a). GPR91 is an orphan GPCR highly expressed in the kidney and shares 33% amino acid identity with GPR99/GPR80 (refs 4, 8). On the basis of their homology with the purinergic receptor P2Y1, nucleotide ligands were predicted for GPR91 and GPR99 (ref. 4). However, the GPR91 ligand activity in pig kidney extracts was resistant to various stringent treatments including alkaline phosphatase, peptidase, and hydrolysis in 6 M HCl at 100 °C. Accordingly, the supposition that GPR91 might be activated by a nucleotide or peptide ligand was unlikely. We purified the natural ligand for GPR91 by ion-exchange, size-exclusion and reversed-phase fast performance liquid chromatography/high-performance liquid chromatography (Fig. 1a).

A major molecular ion $[M + H]^+$ at *m/z* 119.2 was observed by mass spectrometry (Fig. 1b). ^1H NMR analysis revealed a single type of proton in the highly purified GPR91 ligand (Fig. 1c). ^{13}C NMR analysis further suggested the presence of $-CH_2-$ (methylene) and =C=O (carbonyl) groups (Fig. 1d). Combined with mass spectrometry results and the biochemical properties of the ligand, the purified GPR91 ligand was predicted and confirmed to be succinic acid (Fig. 1c, d).

Commercially obtained succinate (the physiological form of succinic acid) increased $[Ca^{2+}]_i$ dose-dependently in the aequorin assay (Fig. 2a). Succinate also activated mouse and rat orthologues of GPR91 (Fig. 2a). The succinate-induced increase in $[Ca^{2+}]_i$ was further confirmed with a fluorimetric imaging plate reader (FLIPR) system in the 293-hGPR91 cell line (293 cells stably expressing human GPR91) (Fig. 2b). The concentration giving half-maximal response (EC_{50}) for succinate-induced activation of human GPR91 was 56 ± 8 μM in the aequorin assay and 28 ± 5 μM in the FLIPR assay. GPR91 was selectively activated by succinate but not by other citric acid cycle intermediates (Supplementary Information) or any of a collection of 800 pharmacologically active compounds and known GPCR ligands tested (data not shown). Furthermore, of 200

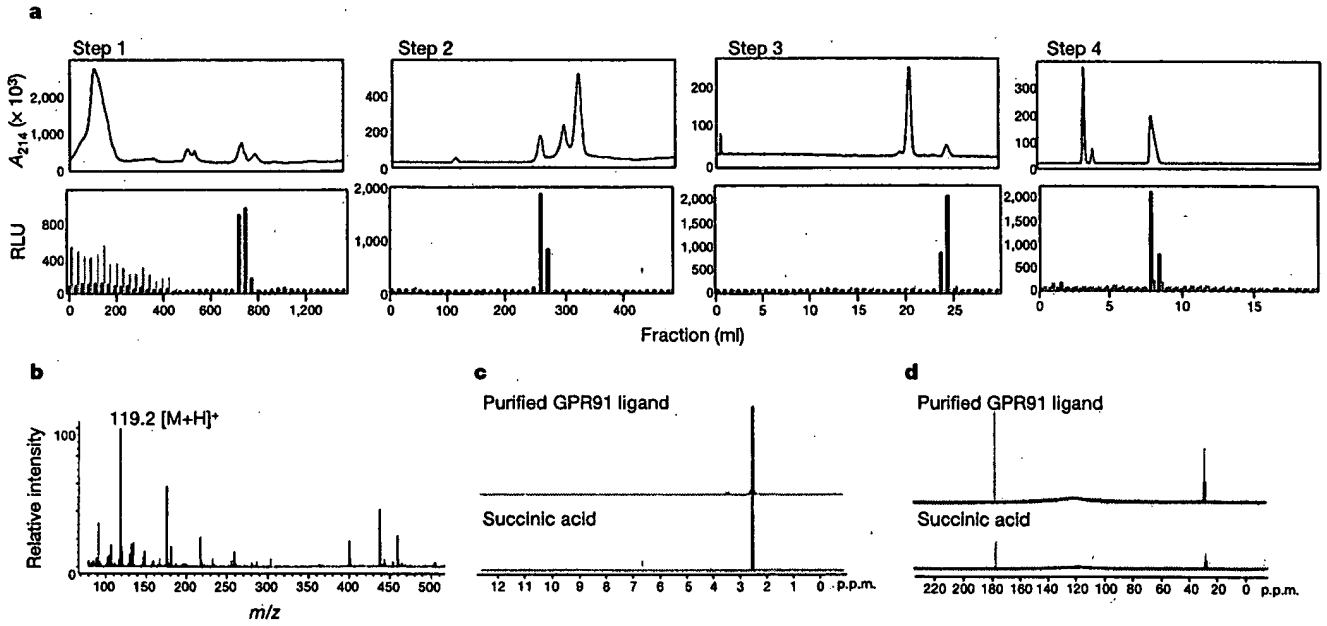

Figure 1 Purification and identification of GPR91 ligand from pig kidney extracts. **a**, Purification of GPR91 ligand through four steps of fast performance liquid chromatography/high-performance liquid chromatography (step 1, anion exchange; step 2, size exclusion; step 3, size exclusion; step 4, reversed phase). The activity of each fraction was assayed by the aequorin assay in CHO cells transfected with GPR91 (black bars) or control plasmid (grey bars). RLU, relative luminescence unit. **b**, Mass spectrometric analysis of the purified GPR91 ligand. **c**, **d**, ^1H NMR (**c**) and ^{13}C NMR (**d**) of the final purified GPR91 ligand: analysis and comparison with succinic acid.

carboxylic acids and structurally related analogues, succinate was found to be the most potent in increasing $[Ca^{2+}]_i$ (a representative subset is summarized in Supplementary Information).

Because GPR99 is the closest homologue of GPR91, we proposed that other citric acid cycle intermediates and carboxylic acids might activate GPR99. We identified α-ketoglutarate as a ligand for human GPR99, with an EC_{50} of 69 ± 11 μM in the aequorin assay (Fig. 2c) and 32 ± 4 μM in the FLIPR assay (Fig. 2d). α-Ketoglutarate also activated mouse GPR99 (Fig. 2c). Neither succinate nor α-ketoglutarate activated calcium mobilization with about 30 GPCRs unrelated to GPR91 and GPR99 (data not shown).

To dissect the signalling pathways of GPR91 and GPR99, various biochemical assays were investigated. Succinate inhibited forskolin-stimulated cAMP production in 293-hGPR91 cells, an effect abolished by preincubation with pertussis toxin (PTX) (Fig. 2e). In addition, succinate induced the accumulation of inositol phosphate in 293-hGPR91 cells (Fig. 2f). The succinate-induced accumulation of inositol phosphate and an increase in $[Ca^{2+}]_i$ were each partly inhibited by pertussis toxin (Fig. 2f and Supplementary Information). Moreover, succinate activated extracellular signal-regulated kinase (Erk) in 293-hGPR91 cells (Supplementary Information). Taken together, these results suggest that GPR91 activation by succinate couples to at least two signalling pathways, a pertussis-toxin-sensitive G_i/G_o pathway and a pertussis-toxin-insensitive G_q pathway. Similarly, α-ketoglutarate stimulated inositol phosphate formation in a GPR99-dependent manner (Fig. 2g). Because GPR99 activation did not affect cAMP levels (data not shown), and both inositol phosphate formation (Fig. 2g) and $[Ca^{2+}]_i$ flux (Supplementary Information) were found to be insensitive to pertussis toxin, GPR99 seems to act exclusively through a G_q-mediated pathway.

Ligand-induced receptor internalization is often characteristic of GPCR activation and signal attenuation[9]. Immunofluorescent staining of cells expressing Flag-tagged GPR91 or GPR99 revealed that both GPR91 and GPR99 were localized to the plasma membrane (Fig. 2h). Ligand stimulation induced the internalization of both receptors (Fig. 2h).

Because the dicarboxylate groups of succinate and α-ketoglutarate are required for the activation of GPR91 and GPR99, we speculated that basic residues of GPR91 and GPR99 might be important in binding dicarboxylate ligands. A partial three-dimensional model of GPR91 was generated. Mutation of Arg 99, His 103, Arg 252 or Arg 281 (Supplementary Information) abolished GPR91 activation by succinate, whereas mutation of His 249, Arg 255 or Tyr 107 had no effect on receptor function (Fig. 3a). Various GPR91 mutants were expressed at similar levels (Fig. 3b) and localized at the plasma membrane (data not shown). All four residues required for GPR91 activation by succinate (Arg 99, His 103, Arg 252 and Arg 281) are directed into the central cavity where *cis*-retinal binds in the rhodopsin structure (Fig. 3c, d). These four positively charged residues cluster together, Arg 99 and His 103 of helix III on one side and Arg 252 of helix VI and Arg 281 of helix VII on the other side,

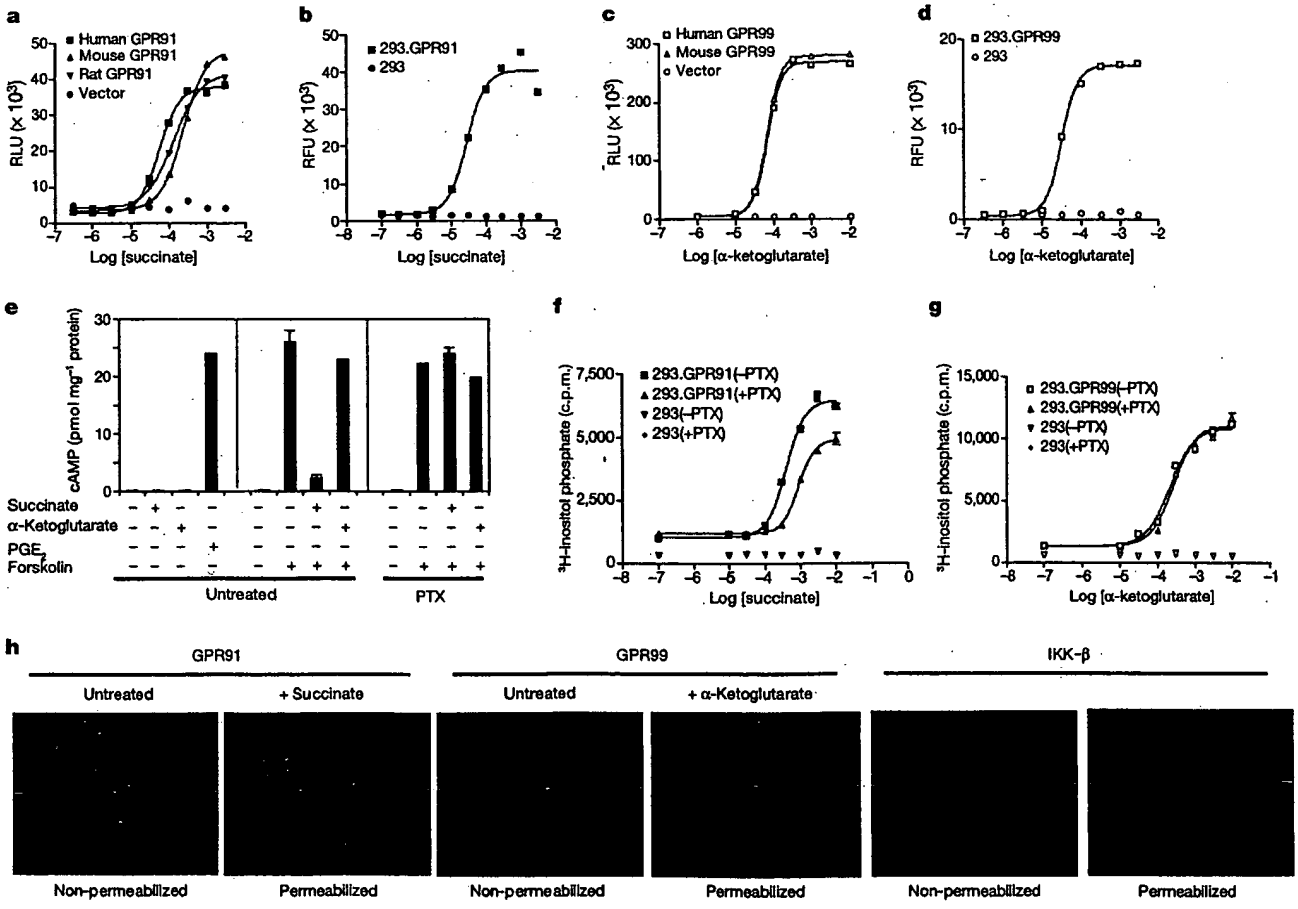

Figure 2 Pharmacological characterization. Data are shown as means ± s.e.m. for three or four determinations. **a**, GPR91 activation by succinate in aequorin assay. **b**, FLIPR assay in 293-hGPR91 cells. RFU, relative fluorescence unit. **c**, **d**, Activation of GPR99 by α-ketoglutarate in aequorin (**c**) and FLIPR assays (**d**). **e**, Effect of succinate (200 μM) on intracellular cAMP in 293-hGPR91 cells. Pertussis toxin and forskolin were included as indicated. Prostaglandin E_2 (PGE_2) was used as a control. **f**, Inositol phosphate formation in 293-hGPR91 cells and the effect of pertussis toxin (PTX). **g**, Inositol phosphate accumulation in 293-hGPR99 cells and the effect of PTX. **h**, Immunofluorescence staining. Plasma membrane localization of the receptors (in non-permeabilized cells) and the internalized vesicles (in permeabilized cells) were visualized by anti-Flag antibody. IKK-β, IκB kinase-β. An intracellular protein IKK-β[30] served as a control.

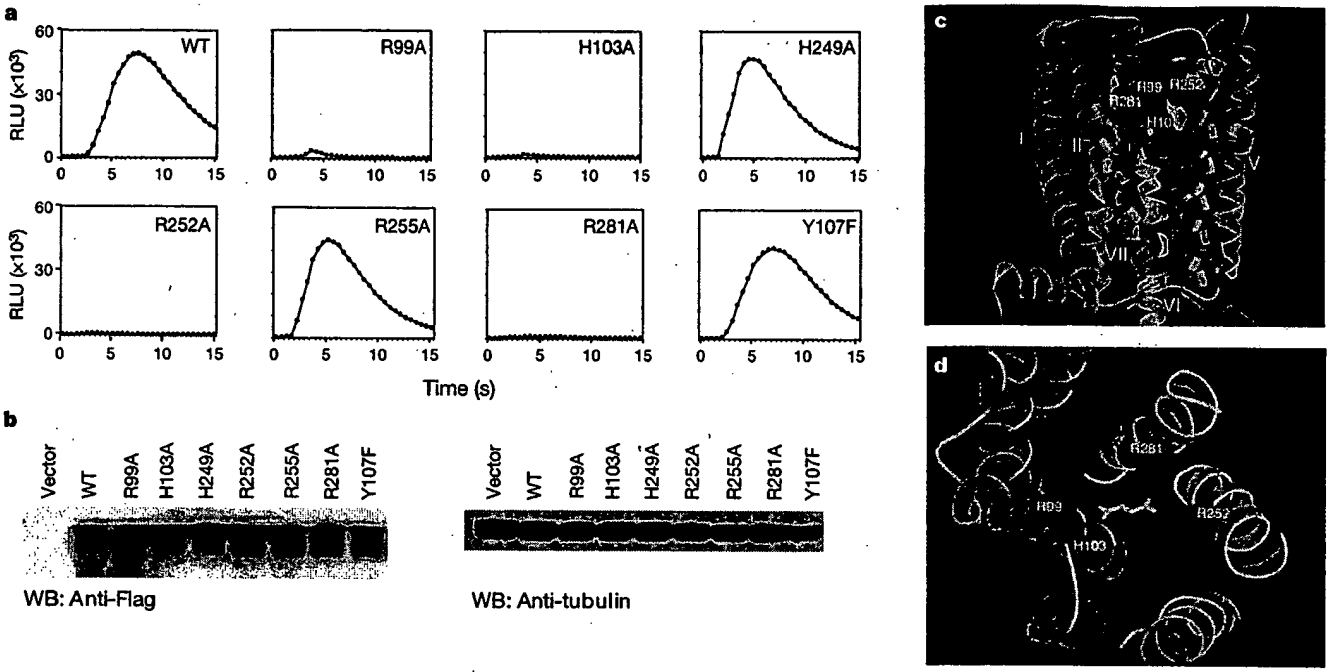

Figure 3 Mutational analysis and predicted three-dimensional model for GPR91. **a**, Activation of wild-type (WT) and mutant GPR91 receptors by succinate (200 μM) in aequorin assay in CHO cells. **b**, The expression of wild-type and mutant GPR91 receptors in CHO cells as determined by western blot (WB) analysis. **c**, Predicted sites for Arg 99, His 103, Arg 252 and Arg 281 in the three-dimensional ribbon presentation of GPR91. The main chains of these four residues are coloured in blue. **d**, Top view from the extracellular side of the four basic residues in the ligand-binding pocket with a succinic acid molecule placed in the cavity.

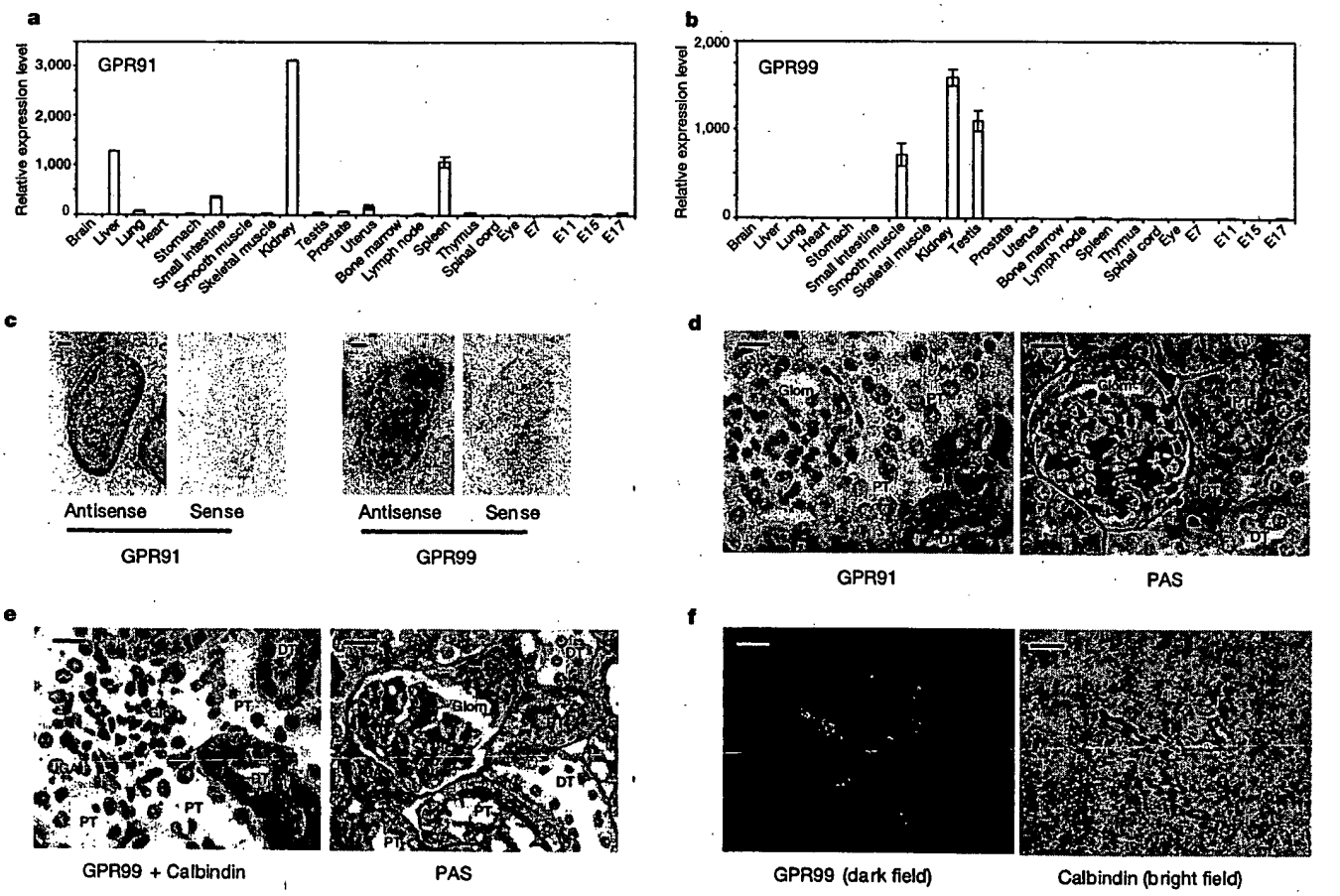

Figure 4 Regional distribution of GPR91 and GPR99. **a**, **b**, Q-RT–PCR analysis of GPR91 (**a**) and GPR99 (**b**) in mouse tissue. Data are shown as means ± s.e.m. for triplicate determinations. **c**, Localization of GPR91 and GPR99 in mouse kidney sections by *in situ* hybridization. Scale bar, 1 mm. **d**, Localization of GPR91 (black grains) in the proximal tubules. PT, proximal tubule; DT, distal tubule; glom, glomerulus. Sections were prestained with anti-calbindin (brown) before hybridizing to GPR91 probe. Periodic acid–Schiff (PAS) staining identified proximal tubules. Scale bar, 25 μm. **e**, **f**, Localization of GPR99 (black grains in **e** and white signals in **f**) in the distal tubules. Scale bar: 25 μm (**e**); 0.25 mm (**f**).

and may provide a unique electrostatic complementary binding environment for succinate (Fig. 3d). A comparison of about 300 family A GPCRs revealed that GPR91 and GPR99 uniquely contain all four basic residues, suggesting that GPR91 and GPR99 are specialized receptors for dicarboxylate ligands.

Expression analysis by quantitative reverse-transcriptase-mediated polymerase chain reaction (Q-RT–PCR) revealed that the GPR91 and GPR99 messenger RNAs were each predominantly expressed in the kidney, with limited expression in other tissues (GPR91 in the liver and the spleen, and GPR99 in the testis and the smooth muscle; Fig. 4a, b and refs 3, 4). *In situ* hybridization experiments indicated specific GPR91 and GPR99 expression in the cortical region of the mouse kidney (Fig. 4c). GPR91 was detected mainly in the proximal tubules, as demonstrated by the presence of a prominent brush border projecting into the lumen on periodic-acid–Schiff-stained adjacent sections (Fig. 4d). GPR91 was also found, to a smaller extent, in the distal tubules (revealed by staining with antibodies against the distal-tubule marker calbindin[10]; Fig. 4d) and the juxtaglomerular apparatus (Supplementary Information). In contrast, GPR99 was found predominantly in the distal tubules (Fig. 4e, f).

Intravenous injection of succinate into Sprague–Dawley rats increased plasma renin activity (Fig. 5a), which is consistent with a previous report that succinate-treated kidney cultures *ex vivo* released renin[11]. Renin is a key enzyme in the renin–angiotensin system, which is essential in blood pressure regulation[12,13]. Cumulative intravenous administration of succinate produced a dose-dependent increase in mean arterial pressure (MAP) (Fig. 5b). Bilateral nephrectomy completely abolished this effect (Fig. 5c), demonstrating the kidney as the primary site of action. To dissect the pharmacological mechanism for succinate-induced hypertension, the angiotensin-converting enzyme inhibitor captopril was used. Pretreatment with captopril significantly attenuated the hypertensive effect of succinate (Fig. 5d), indicating that the angiotensin-converting enzyme product(s) might mediate hypertension induced by succinate. In contrast, neither nephrectomy nor captopril treatment had any effect on angiotensin II-induced hypertension (Fig. 5c, d).

To further explain the mechanisms underlying succinate-induced hypertension, we generated GPR91-deficient mice by homologous recombination (Fig. 5e, f). Mice homozygous for the targeted allele of GPR91 are viable and have no discernable phenotype. The systolic blood pressures and heart rates were similar in unanaesthetized wild-type and GPR91-deficient mice (Fig. 5g; systolic blood pressures were 129.5 ± 1.5 mmHg for wild-type and 131.2 ± 2.4 mmHg for GPR91-deficient mice; heart rates were 613 ± 12.4 beats min^{-1} for wild-type and 606 ± 11.4 beats min^{-1} for GPR91-deficient mice). However, succinate could no longer induce hypertension in GPR91-deficient mice (Fig. 5h, i). In contrast, angiotensin II increased blood pressure similarly in wild-type and

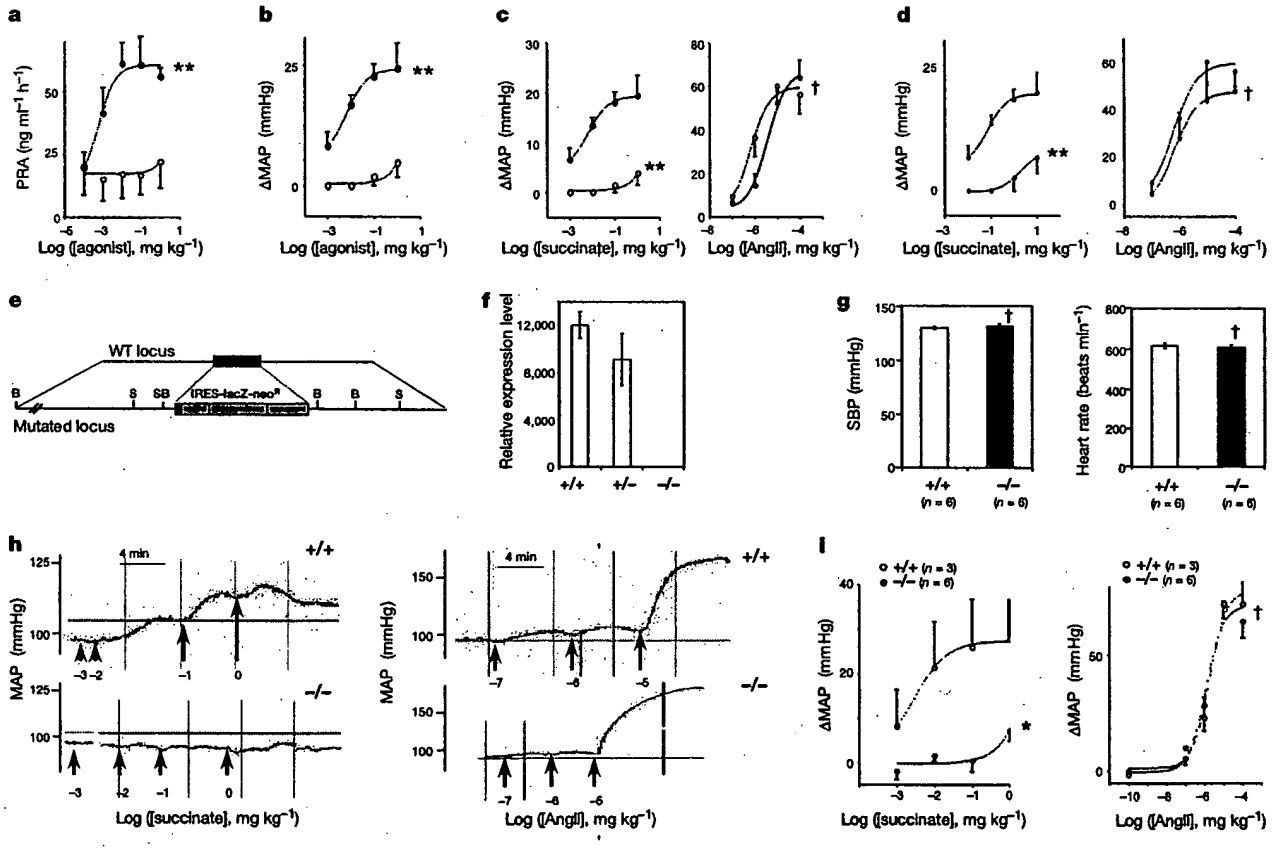

Figure 5 The pro-hypertensive effect of succinate and GPR91. All results are shown as mean ± s.e.m. Single asterisk, $P < 0.05$; double asterisk, $P < 0.01$; dagger, $P > 0.05$ (two-way analysis of variance or *t*-test). **a**, Plasma renin activity (PRA) in Sprague–Dawley rats by intravenous succinate infusion. Filled circles, succinate ($n = 6$); open circles, α-ketoglutarate ($n = 6$). **b**, MAP in Sprague–Dawley rats by intravenous succinate infusion. Values are the change in MAP (ΔMAP, mmHg) for each test group above its vehicle-injected control group. Filled circles, succinate ($n = 8$); open circles, α-ketoglutarate ($n = 6$). **c**, Effect of bilateral nephrectomy. Filled circles, kidney intact ($n = 6$); open circles, bilateral nephrectomy ($n = 4$). **d**, Effect of captopril. Filled circles, captopril present ($n = 4$); open circles, captopril absent ($n = 6$). **e**, Schematic map of the GPR91 wild-type (WT) locus and the inactivated GPR91 allele. Black box, open reading frame of GPR91 gene; S, *Spe*I; B, *Bam*HI; lacZ, β-galactosidase expression cassette; neo^R, neomycin resistance gene cassette. **f**, Q-RT–PCR analysis of GPR91 mRNA in kidneys of wild type (+/+), GPR91-heterozygous (+/−) and GPR91-deficient (−/−) mice. **g**, Systolic blood pressure and heart rate of unanaesthetized wild-type and GPR91-deficient mice. **h**, **i**, MAP tracing measurements (**h**) and dose–response curves (**i**) of wild-type and GPR91-deficient mice administered intravenously with succinate (left panels) or angiotensin II (AngII) (right panels).

GPR91-deficient mice (Fig. 5h, i), indicating that the hypertensive effect of succinate might be mediated by GPR91.

Succinate and α-ketoglutarate are normally present in mitochondria, but are found in circulation with mean plasma levels of about 5 μM for succinate and about 25 μM for α-ketoglutarate[14–16]. Succinate is known to increase the reabsorption of phosphate and glucose into the proximal tubule[17] and to stimulate gluconeogenesis[18]. Accumulation of extracellular succinate is observed in physiological and/or pathophysiological states linked to the deterioration of blood supply, such as ischaemia[19,20]. It is known that the renovascular hypertension is often associated with restricted blood supply to the kidney owing to the obstruction of renal arteries in diseases such as atherosclerosis[5]. It is possible that the accumulation of succinate in ischaemic conditions might contribute to stenosis-associated hypertension through the activation of GPR91. Although we have shown that the hypertensive effect of succinate is mediated by GPR91 through the activation of the renin–angiotensin system, the molecular mechanism of renin release by succinate is not clear. Because GPR91 is a G_q- and G_i-coupled receptor, it is unlikely that GPR91 functions directly on the juxtaglomerular cells to stimulate renin release, which is usually triggered by G_s activation[21]. It remains to be investigated whether sympathetic nerve activity, prostaglandins or nitric oxide[22] might provide an indirect mechanism for succinate-induced renin release. The presence of GPR91 in the liver and the spleen might also have functions in mediating these effects.

Thus, we have identified succinate as the natural ligand for GPR91, and α-ketoglutarate as the ligand for the homologous receptor GPR99. We have shown that intermediates in the citric acid cycle function as signalling molecules, engendering renewed interest in a biochemical pathway discovered more than 60 years ago. We have provided evidence that the pro-hypertensive effect of succinate is mediated by GPR91 and involves the renin–angiotensin system. GPR91 activation by succinate might therefore provide a link between energy homeostasis/metabolic status and haemodynamic regulation. The finding that intermediates in the citric acid cycle are GPCR ligands should facilitate the understanding of molecular links of the citric acid cycle to metabolic diseases, such as hypertension, atherosclerosis and diabetes, and the design of novel drugs with GPR91 and GPR99 as molecular targets. □

Methods

Purification of GPR91 ligand

Pig kidneys (6 kg; Pel-Freeze) were homogenized and extracted in ethanol/water/acetic acid (50/46/4, by vol.). The supernatant was freeze-dried, resuspended in 10 mM K_2HPO_4 pH 8.0, applied to an XK50/20 Q Sepharose anion-exchange column (Amersham), and developed with a 0–0.25 M NaCl linear gradient. The active fractions were subjected to three more steps of purification: a Hiload 26/60 Superdex30 size-exclusion column (developed in Hank's buffered saline at pH 7.35); a Superdexpeptide HR10/30 size-exclusion column (developed in 0.1% trifluoroacetic acid/water); and a C_{18} 4.6 mm × 250 mm reverse-phase column (developed in 0.1% trifluoroacetic acid /water). The active fractions from the final purification step were freeze-dried and dissolved in ^2H_2O for NMR and mass spectrometry analysis.

NMR and mass spectrometry

The ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX700 spectrometer (Bruker Analytik GmbH) at 700 and 176 MHz, respectively. The mass spectrum was obtained on an HP 1100MSD spectrometer (Agilent Technologies) with electrospray ionization in positive detection mode. A full-scan mass spectrum covering the *m/z* range 50–500 was acquired.

Cell culture and signalling assays

Chinese hamster ovary (CHO) cells were transiently transfected with 5 μg GPCR and 5 μg aequorin reporter gene[7] in 100-mm dishes as described[23]. Aequorin luminescence was recorded with a Microlumat (Berthold). FLIPR assays were performed on FLIPR384 with a FLIPR calcium assay kit (Molecular Devices). Inositol phosphate formation was assayed as the incorporation of ^3H-*myo*-inositol as described[23,24]. Cells were stimulated with compounds in the presence of 10 mM LiCl for 40 min at 37 °C before the addition of 20 mM formic acid. cAMP assay was performed with the cAMP-Screen system (Applied Biosystems). Cells were stimulated for 20 min with 10 μM forskolin before treatment with compounds for a further 20 min at 37 °C. Anti-phospho-Erk and anti-Erk monoclonal antibodies were obtained from New England Biolabs. Anti-Flag and anti-tubulin antibodies were obtained from Sigma. Western blots were detected with the ECL Plus detection system (Amersham). For some studies, 100 ng ml^{-1} pertussis toxin (Calbiochem) was incubated with cells for 16 h.

Immunofluorescence staining

Human embryonic kidney 293 cells transfected with Flag-tagged GPR91 or GPR99 were plated on poly-(D-lysine)-treated slides. Cell-surface staining and ligand-induced internalization was performed as described[9]. Internalization of GPR91 or GPR99 was induced for 10 min by succinate (200 μM) or α-ketoglutarate (300 μM) at 37 °C.

In situ hybridization, immunohistochemistry and expression analysis

These were performed as described[10,25]. [^{33}P]UTP-labelled antisense or sense RNA probes for mouse GPR91, GPR99 or renin were hybridized to paraformaldehyde-fixed, paraffin-embedded mouse kidney sections. For periodic acid–Schiff and renin staining, adjacent mouse kidney sections were used. For co-localization with calbindin on the same tissue section, slides were incubated with anti-calbindin antibody (Sigma) and developed with Vectastain avidin–biotin-complex kit (Vector Lab) with 3,3′-diaminobenzidine as substrate before hybridization *in situ*. Sections were counterstained with haematoxylin or toluidine blue. Q-RT–PCR was performed on an ABI Prism 7700 sequence detector using Taqman Gold kit (Applied Biosystems). Ratios of GPR91 or GPR99 to glyceraldehyde-3-phosphate dehydrogenase mRNA were calculated. Primer and probe sequences for GPR91 and GPR99 are available from the authors on request.

Receptor model

The published bovine rhodopsin structure was used as a template for generating a partial GPR91 structure model[26]. The GPR91 primary sequence was aligned to bovine rhodopsin (PDB code 1F88; Supplementary Information), and the three-dimensional homology model was generated with the Modeller program.

In vivo experiments

All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee. Animals were housed in a temperature-controlled room under a 12 h light–dark cycle with water and standard rodent chow available *ad libitum*. Blood pressure was measured in unanaesthetized animals by the tail-cuff method or in pentobarbitol-anaesthetized animals by the intra-arterial method for those involving chemical administration[27,28]. Male Sprague–Dawley rats (about 300 g; Harlan) or mice (about 30 g; Lexicon) were used. Systolic blood pressure was recorded in mice for 5 days; at least 20 measurements were made daily with a BP-2000 blood pressure analysis tail-cuff system (Visitech Systems). The mean systolic blood pressure and pulse rate were taken for each animal. Intra-arterial measurement of blood pressure was achieved with a polyethylene PE50 (for rats) or a stretched PE10 (for mice) catheter implanted into the carotid artery, with another implanted into the jugular vein before the experiments. The artery catheter was connected to a high-sensitivity isometric transducer (Harvard Apparatus) for continuous recording of blood pressure. MAP data were acquired by Ponemch Physiology Platform (Gould Instrument). The venous catheter was used for chemical administration. Bilateral nephrectomy was performed as described[29]. Captopril (10 mg kg^{-1}) was given as a bolus injection 30 min before the administration of succinate. Plasma renin activity was determined by radioimmunoassay as the rate of angiotensin I generation (DiaSorin). GPR91-deficient mice were generated by homologous recombination in embryonic stem cells (Lexicon). Results are expressed as means ± s.e.m. Statistical significance was evaluated by Student's *t*-test or two-way analysis of variance, followed by Dunnett's *post-hoc* test. Differences resulting in $P < 0.05$ were considered significant.

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Aquaporin-0 membrane junctions reveal the structure of a closed water pore

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The lens-specific water pore aquaporin-0 (AQP0) is the only aquaporin known to form membrane junctions *in vivo*[1]. We show here that AQP0 from the lens core, containing some carboxy-terminally cleaved AQP0[2,3], forms double-layered crystals that recapitulate *in vivo* junctions. We present the structure of the AQP0 membrane junction as determined by electron crystallography. The junction is formed by three localized interactions between AQP0 molecules in adjoining membranes, mainly mediated by proline residues conserved in AQP0s from different species but not present in most other aquaporins. Whereas all previously determined aquaporin structures show the pore in an open conformation[4–9], the water pore is closed in AQP0 junctions. The water pathway in AQP0 also contains an additional pore constriction, not seen in other known aquaporin structures[4–9], which may be responsible for pore gating.

AQP0 is a member of the aquaporin family, members of which form pores that are either highly selective for water (aquaporins) or also permeable to other small neutral solutes such as glycerol (aquaglyceroporins) (reviewed in ref. 10). To date, the atomic structures of three aquaporins have been determined (AQP1[4–6], GlpF[7,8] and AQPZ[9]). Sequence alignment shows AQP0 to be closely related to the pure water channel AQP1 (43.6% identity, 62.6% similarity). The presence of His 172, a residue conserved only in aquaporins but substituted in aquaglyceroporins, also suggests that AQP0 forms a pure water pore. AQP0 water permeability at neutral pH is approximately 40 times lower than that of AQP1[11], but AQP0 water conductance doubles under mildly acidic conditions[12]. In the case of aquaporins in plant roots, a pH-dependent closure of the water pores has been reported[13]. Thus, evidence suggests that certain aquaporin pores are gated.

AQP0 water pores are considered essential for the lens microcirculation system, proposed to supply deeper-lying fibre cells with nutrients and to clear waste products[14,15]. Unlike all other aquaporins, AQP0 is also present in membrane junctions. It is particularly enriched in the 11–13 nm thin junctions between lens fibre cells, that feature square AQP0 arrays[1]. Atomic force microscopy analysis of *in vitro* reconstituted AQP0 two-dimensional crystals demonstrated these crystals to be double-layered[16].

Using AQP0 from the core of sheep lenses, where some of the AQP0 is proteolytically cleaved near the C terminus at various sites in an age-dependent manner[2,3], we reproduced the double-layered two-dimensional crystals[16,17]. When core AQP0 was reconstituted at a lipid-to-protein ratio of 0.25 (w/w), large membrane sheets formed (>6 μm) that in some cases showed two parallel edges, revealing them to be double-layered (Fig. 1a). The crystals showing $p422$ symmetry had lattice constants of $a = b = 65.5$ Å and a thickness of 11 nm (Fig. 2a), the same dimensions as thin junctions in the lens[1]. Double-layered AQP0 two-dimensional crystals are therefore likely to recapitulate thin lens fibre cell junctions.

Electron diffraction analysis of AQP0 crystals (tilted to an angle of up to 70°) produced strong diffraction spots to 3 Å resolution in all directions (Fig. 1b, c; the electron crystallographic data are summarized in Table 1). As the crystal structure of the homologous bovine AQP1 was available[5], we determined the structure of the AQP0 membrane junction by molecular replacement, thus avoiding the cumbersome and time-consuming process of collecting high-resolution images of tilted specimens. Sequencing of cloned sheep AQP0 showed an identical amino acid sequence to bovine AQP0[18], with the exception of three conservative (S20T, M90V and S240T) and one non-conservative substitutions (C14F).

Our model (Fig. 2a) shows unique features that enable AQP0 to form membrane junction interactions. These differ from those previously suggested based on atomic force microscopy data[16]. The extracellular surface of AQP0 is rather flat and the interactions are mediated by direct contacts of the corresponding loops in the opposing AQP0 molecules (Fig. 2a). Loop C, connecting α-helices three and four, is significantly shorter than in AQP1 and GlpF. The shortened loop C (also seen in AQP2, AQP5, AQP6 and AQP8) is crucial for the formation of the very tight AQP0 junction, as it allows three specific interactions to be formed that are mediated almost exclusively by proline residues.

The most striking interaction involves Pro 38, in extracellular loop A. The proline residues (Pro 38) from eight symmetry-related AQP0 molecules in the stacked tetramers come together to form a

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